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Generation of reactive oxygen species by a novel anthraquinone derivative in the cyanobacterium *Planktothrix perornata* (Skuja)

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Abstract

A water-soluble anthraquinone derivative (2-[methylamino-*N*-(1′-methylethyl)]-9,10-anthraquinone monophosphate), previously found to be selectively toxic towards *Planktothrix perornata* at submicromolar concentrations, was studied to determine its toxic mode of action towards this cyanobacterium. Chlorophyll fluorescence was monitored as an indicator of photosynthetic efficiency, and measurement of reactive oxygen species (ROS) was performed using the ROS-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate. The effects of the herbicide paraquat (a ROS generator) as well as ascorbate and α-tocopherol (ROS scavengers) on ROS formation by *P. perornata* were studied. Also, the effects of different concentrations of ascorbate, α-tocopherol, and the herbicide diuron on reducing the toxicity of the water-soluble anthraquinone derivative towards *P. perornata* were determined. Our results indicate that the water-soluble anthraquinone derivative does not inhibit photosynthetic electron transport directly, but does generate ROS at levels that may cause toxicity towards *P. perornata*.

Keywords: Anthraquinone; Catfish; Cyanobacteria; 2-Methylisoborneol; Reactive oxygen species

1. Introduction

The largest type of aquaculture in the United States is farm-raised channel catfish (*Ictalurus punctatus*) and most channel catfish production occurs in the state of Mississippi. Other than disease-related problems, ''off-flavor'' in cultured catfish results in the largest economic losses to catfish producers in the southeastern United States of $20–30 million annually. The most common off-flavor is ''musty'' and is caused by absorption of the compound 2-methylisoborneol (MIB) into the flesh

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of the farm-raised catfish [1]. Producers must hold catfish determined to be off-flavor by flavor analysis until they lose the musty taint. These delays in harvest cause economic burdens to producers due to additional feed costs, forfeiture of income from foregone sales, interruption of cash flow, and loss of held catfish to disease, poor water quality, and bird depredation [2].

Certain species of cyanobacteria (blue-green algae) produce MIB, and the cyanobacterium "*Planktothrix perornata*" (Skuja) is attributed as the main cause of MIB-related off-flavor in cultured catfish in west Mississippi [3]. This cyanobacterium was designated *Oscillatoria perornata f. attenuata* [4] based upon the descriptions by Skuja [5] and recently reassigned as *Planktothrix perornata f. attenuata* (Skuja) [6]. Once a bloom of *P. perornata* occurs in a catfish pond, all of the catfish in that pond are likely to be tainted with musty off-flavor.

One current management approach for controlling musty off-flavor in cultured catfish is the application of algicides to ponds to kill the odor-producing cyanobacteria. The algicides currently being used by catfish farmers are 3-[3,4-dichlorophenyl]-1,1-dimethylurea (diuron) and copper-based products such as copper sulfate and chelated-copper compounds. These herbicides have several negative qualities including broad-spectrum toxicity towards beneficial phytoplankton in the catfish ponds and high persistence in the pond environment [2,7]. Diuron can be absorbed into the flesh of catfish and, therefore, producers must follow strict guidelines for its application to catfish ponds [8]. In addition, diuron has been approved by the United States Environmental Protection Agency (USEPA) for use in catfish aquaculture ponds as a selective algicide under a section 18 emergency exemption which requires annual review for renewal. Such renewal is not guaranteed in the future due to USEPA reregistration requirements for diuron. The discovery of environmentally safe selective algicides would greatly benefit the United States catfish industry.

Previous research by Schrader et al. [9] found anthraquinone to be a promising natural selective algicide (cyanobactericide), and, subsequently, a novel water-soluble derivative of anthraquinone, 2-[methylamino-*N*-(1′-methylethyl)]-9,10-anthraquinone monophosphate (Fig. 1), was developed to selectively kill *P. perornata* in catfish ponds [10]. Schrader et al. [10] found that the water-soluble anthraquinone derivative (WSAD) was selectively toxic towards *P. perornata* with a lowest-complete-inhibition concentration (LCIC) of 0.1 μM compared to the LCIC for *Selenastrum capricornutum* of 100 μM in microplate bioassays. Pond efficacy studies determined that 0.3 μM of WSAD was effective in reducing the abundance of *P. perornata* in catfish aquaculture ponds [10]. Due to its high toxicity towards *P. perornata*, WSAD appears to be promising as a selective algicide to control musty off-flavor in catfish aquaculture ponds. Studies to determine the toxic mode of action of WSAD towards *P. perornata* would be helpful to identify future promising compounds for screening as selective algicides based upon their chemical structure and/or known modes of action. Other studies found that 9,10-anthraquinone inhibits photosynthetic electron transport at 1 μM in *P. perornata* and, thereby, adversely affects its growth [4]. In this study, we monitored chlorophyll fluorescence, the production of reactive oxygen species (ROS), and the effects of the ROS scavengers ascorbate and α-tocopherol (vitamin E) on WSAD toxicity towards *P. perornata* to help determine the toxic mode of action of WSAD towards *P. perornata*.

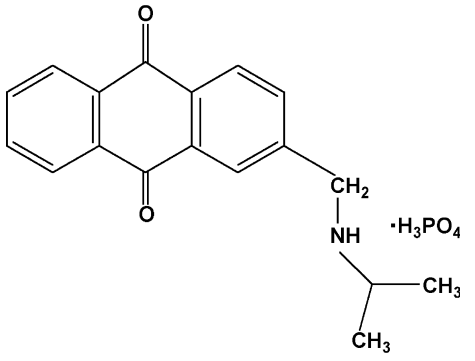

Fig. 1. Water-soluble anthraquinone derivative (WSAD).

2. Materials and methods

2.1. Culture conditions

Cultures of *P. perornata* (isolated from a Mississippi catfish pond) and the unicellular green alga *S. capricornutum* (Printz) were maintained in a continuous, steady state to provide a source of cells growing at a constant rate [11]. *S. capricornutum* is also found in catfish ponds in Mississippi and was tested for ROS production since it is less sensitive to WSAD than *P. perornata* according to microplate bioassays [10].

2.2. Measurement of photosynthetic electron transport

The first set of experiments used to determine the toxic mode of action of WSAD towards *P. perornata* were similar to those used by Schrader et al. [4] for testing anthraquinone. Stock solutions of WSAD were made in 100% ethanol. Cells of *P. perornata* obtained from continuous cultures were incubated under red light for 30 min at 27 °C in the presence of different inhibitors. All of the herbicides used in this study were technical grade quality, and test compound stock solutions were prepared with compensations for purity. Concentrations of WSAD (99% pure), atrazine (98% pure) [6-chloro-*N*-ethyl-*N*′-(1-methyethyl)-1,3,5-triazine-2,4-diamine; Chem Service, West Chester, PA, USA], and paraquat (99% pure) (1,1′-dimethyl-4,4′-bipyridinium tetrahydrate; Chem Service, West Chester, PA, USA) ranged from 0.003 to 10 μM. Anthraquinone-2-carboxylate (98% pure) (Sigma–Aldrich, St. Louis, MO, USA), the primary breakdown product of WSAD (Schrader and Nanayakkara; unpublished observations) was tested between 0.3 and 10 μM. Control treatments contained the same concentration of ethanol as treated samples (3%). Quantum yield (Y) of photosynthesis was monitored with a pulse-modulated fluorometer (Opti-Science, Model OS5-FL, Tyngsboro, MA) as described before [4]. There were three replicates at each concentration and the experiment was repeated independently.

2.3. Detection of reactive oxygen species

The method of He and Häder [12] was used to monitor the production of ROS in vivo in logarithmically growing cultures of *P. perornata* and *S. capricornutum*, (3–4 and 4–5 μg chlorophyll *a* ml^{-1}, respectively). Culture material obtained from continuous cultures of *P. perornata* and *S. capricornutum* were placed in 100-ml batch culture bottles (50 ml of culture per bottle). Two concentrations of 0.5 and 1.0 μM of WSAD were tested by adding 125 and 250 μl, respectively, from a 200.0 μM stock solution of WSAD in deionized water. The appropriate amount (125 or 250 μl) of deionized water was added to controls. The fluorometric probe 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA; Sigma–Aldrich, St. Louis, MO, USA) was added (final concentration of 5.0 μM from a stock solution of 2.0 mM) under low-intensity green light, and the cultures were immediately placed in the dark at room temperature and air was bubbled through them. Modified BG-11 medium [13], used to culture *P. perornata* and *S. capricornutum*, was also tested for ROS to measure "background" ROS formation. The nonpolar compound DCHF-DA is converted to 2′,7′-dichlorodihydrofluorescein (DCHF) by esterases once it is inside the cell, and DCHF is rapidly oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular ROS [12]. Because DCHF-DA autooxidizes readily, concentrations of WSAD higher than the LCIC of 0.1 μM for *P. perornata* were used in this study to permit more rapid formation of ROS.

After the addition of DCHF-DA, samples (5 ml) from each culture bottle were removed under green light (to help prevent autooxidation of DCHF-DA that can occur in the presence of ultraviolet irradiation or photosynthetically active radiation) at 1, 2, and 4 h, and measured with a spectrofluorometer (RF-5301PC, Shimadzu, Kyoto, Japan) at room temperature, with an excitation wavelength of 485 nm. Fluorescence intensity was measured at an emission wavelength of 520 nm to determine relative ROS production. The results were corrected by subtracting the fluorescence of the BG-11 medium. Each experiment was repeated three times.

The same procedures used for testing WSAD were used with experiments measuring ROS production after the addition of the herbicide paraquat, anthraquinone-2-carboxylate, and the antioxidants ascorbate (99% pure) (Sigma–Aldrich, St. Louis, MO, USA) and α-tocopherol (95% pure) (Sigma–Aldrich, St. Louis, MO, USA). In separate experiments, 0.5 and 1.0 μM of paraquat (in deionized water) and 1.0 μM anthraquinone-2-carboxylate [in 100% (v/v) ethanol] were tested. The appropriate amount of deionized water and ethanol were added to controls for the paraquat and anthraquinone-2-carboxylate experiments, respectively. In another set of experiments, final test concentrations (10.0, 200.0, and 500.0 μM) of ascorbate dissolved in deionized water were added to cultures separately that received 1.0 μM WSAD while controls contained only 1.0 μM WSAD. Separately, final test concentrations of α-tocopherol (10.0, 200.0, and 500.0 μM) dissolved in 100% methanol were placed in batch culture bottles and the methanol was allowed to evaporate completely before culture of *P. perornata* was added (50 ml of culture per bottle). Each experiment was repeated three times.

2.4. Effects of ascorbate and α-tocopherol on WSAD toxicity

The effects of different concentrations of the ROS scavengers ascorbate and α-tocopherol on the toxicity of WSAD towards *P. perornata* were studied. The 96-well microplate bioassay of Schrader et al. [11] was used. Initially, cells of *P. perornata* from a continuous culture system were added to the microplate wells (180 μl per well). Appropriate amounts of WSAD were added to the microplate wells to yield final concentrations of 0.01, 0.1, and 0.5 μM WSAD (three replicates per concentration) while final concentrations of 1.0, 10.0, 100.0, 200.0, 500.0, and 1000.0 μM ascorbate (three replicates for each concentration of WSAD) were added to wells. Controls did not have ascorbate added to them. Microplates were placed in a Percival Scientific Model I-36LL growth chamber (Percival Scientific, Boone, IA, USA) at 28–30 °C and illuminated continuously by overhead fluorescent lamps (20 W) at a light intensity of 18–24 μE m^{-2} s^{-1}. The absorbance of each well was measured daily for three days at 650 nm using a Packard model SpectraCount microplate photometer (Packard Instrument, Meriden, CT, USA). Mean absorbance readings were graphed and compared to determine if ascorbate mitigated the WSAD toxicity towards *P. perornata*. Each experiment was repeated twice. The same procedures were used to test the effects of α-tocopherol. Methanol was used to dissolve α-tocopherol, and 10 μl of appropriate stock solutions of α-tocopherol was added to the wells to which culture material of *P. perornata* was added only after the methanol had completely evaporated.

3. Results and discussion

Previous research [4] found that anthraquinone (insoluble in water) inhibits photosynthetic electron transport at 1.0 μM in *P. perornata* and, thereby, adversely affects its growth at that concentration [9]. In an effort to develop selective algicides from natural product origin for use in catfish aquaculture ponds, anthraquinone was modified to make it more water-soluble (Fig. 1) [10]. Since WSAD is derived from anthraquinone, it was thought to have a mechanism of action similar to the parent compound. However, the inhibitory activity of WSAD on quantum yield (IC_{50} = 800.0 nM) (Fig. 2) was much less than its efficacy in laboratory bioassays (IC_{50} = 6.3 nM) [10]. Its inhibitory activity against photosystem II was also much lower than that observed with atrazine, a known photosystem II inhibitor, or with paraquat, a known diverter of electrons in photosystem I (Fig. 2). Anthraquinone-2-carboxylate had the least amount of inhibitory activity on photosynthesis. The lack of correlation between the concentrations of WSAD required for *P. perornata* growth inhibition and the level required for inhibition of photosynthesis suggested that this natural product-derived algicide inhibited a different target site.

In our study, the ROS measured by DCF fluorescence included only the ROS produced during respiration since cultures were kept in the dark (i.e., no oxidative stress from light irradiation

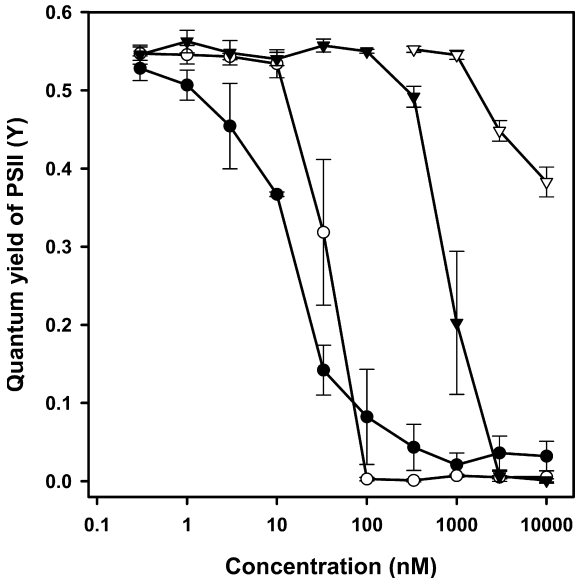

Fig. 2. Effect of atrazine (●), paraquat (○), WSAD (▼), and anthraquinone-2-carboxylate (▽) on the quantum yield of *P. perornata*. Error bars represent ±1 SD.

resulting in additional ROS production). The ROS production by *P. perornata* treated with WSAD at 0.5 and 1.0 μM was higher (approximately 2× after 1 h) than controls (Fig. 3). At 4 h after treatment with WSAD, ROS production was increasing at a greater rate (approximately 3× higher) compared to controls in *P. perornata*. Since DCHF-DA can penetrate cyanobacterial cells to a greater extent than eukaryotic cells due to the presence of cell walls [12], a direct comparison of the DCF fluorescence results between *P. perornata* and *S. capricornutum* cannot be made. There was no difference between ROS production in controls and cultures of *S. capricornutum* receiving 0.5 μM of WSAD (Fig. 3). Only at 4 h after the addition of 1.0 μM of WSAD was ROS production higher in treated cultures of *S. capricornutum* compared to controls. The difference in ROS production between *P. perornata* and *S. capricornutum* may be due in part to differential uptake of WSAD. Based upon microplate bioassays, concentrations of 0.5 and 1.0 μM

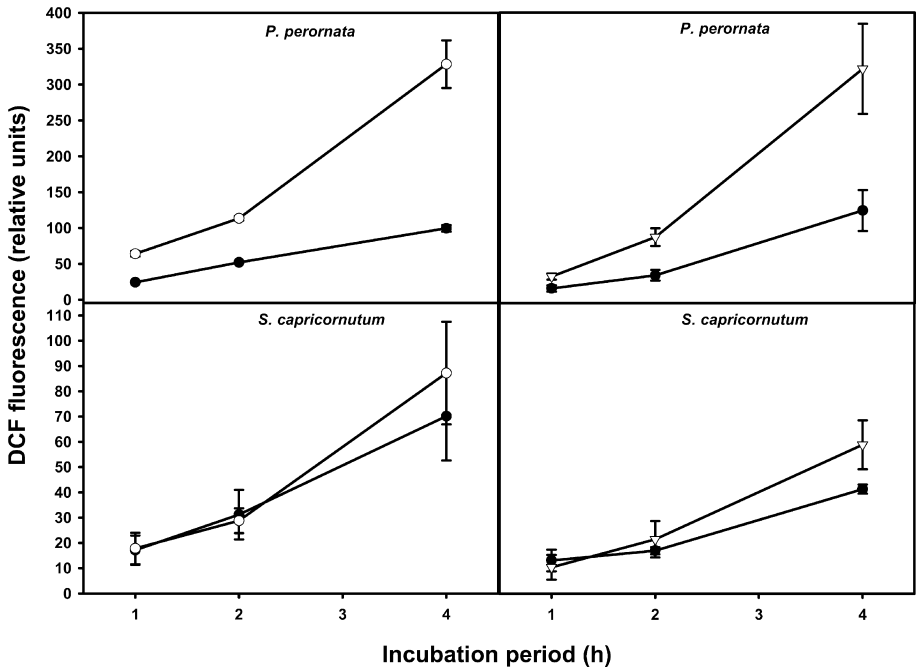

Fig. 3. Effect of 0.5 μM (○) and 1.0 μM (▽) WSAD on ROS production in cultures of *P. perornata* and *S. capricornutum* compared to controls (●) in which no WSAD was added. Error bars represent ±1 SD.

of WSAD were toxic towards *P. perornata*, but not *S. capricornutum* [10].

Paraquat increased ROS formation significantly in cultures of *P. perornata* and *S. capricornutum* compared to controls (Fig. 4). The results of ROS formation from the addition of 0.5 and 1.0 μM paraquat to cultures of *P. perornata* were similar to those results of WSAD. Based upon DCF fluorescence, approximately the same amount of ROS was generated in *P. perornata* by the addition of 0.5 and 1.0 μM of paraquat (Fig. 4) compared to addition of the same concentrations of WSAD (Fig. 3). Paraquat at 1.0 μM generated more ROS in cultures of *S. capricornutum* compared to 1.0 μM of WSAD (Figs. 3 and 4, respectively). Paraquat accepts electrons from photosystem I and reacts with molecular oxygen to form a superoxide radical anion that in subsequent reactions forms ROS such as hydrogen peroxide and hydroxyl radicals [14]. The generation of ROS leads to lipid peroxidation and the destruction of cellular enzymes, membrane fatty acids, and chlorophyll [14,15].

The addition of 200.0 and 500.0 μM ascorbate to cultures containing 1.0 μM of WSAD decreased ROS formation in both cultures as detected by DCF fluorescence (Fig. 5). However, 10.0 μM ascorbate did not reduce ROS production in cultures of *P. perornata* (Fig. 5A), and, in cultures of *S. capricornutum*, 10.0 and 200.0 μM ascorbate (Figs. 5B and D, respectively) did not reduce ROS production compared to those to which 500.0 ascorbate was added (Fig. 5F). Ascorbate is a natural antioxidant that scavenges ROS effectively in combination with ascorbate peroxidase in some cyanobacteria and in eukaryotic algae [16]. Our results suggest the presence of ascorbate peroxidase within the cells of *P. perornata*, though studies confirming ascorbate peroxidase activity are needed for verification.

α-Tocopherol was found to reduce ROS production in cultures of *P. perornata* and *S. capricornutum* at the lowest concentration tested (10.0 μM) (Figs. 6A and B, respectively) as well as the higher concentrations tested (200.0 and 500.0 μM) (Figs. 6C–F). Compared to ascorbate,

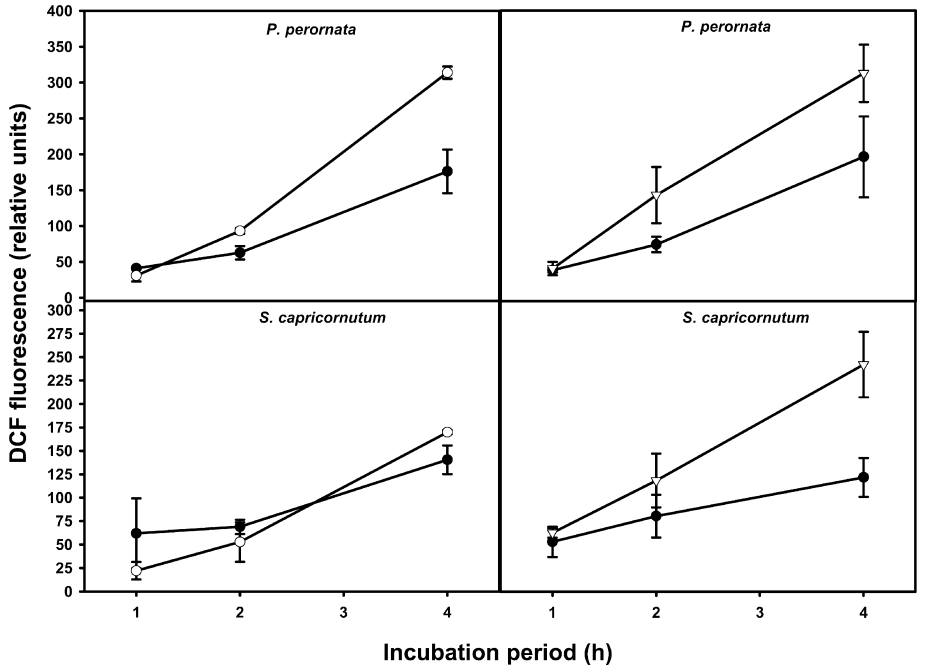

Fig. 4. Effect of 0.5 μM (○) and 1.0 μM (▽) paraquat on ROS production in cultures of *P. perornata* and *S. capricornutum* compared to controls (●) in which no WSAD was added. Error bars represent ±1 SD.

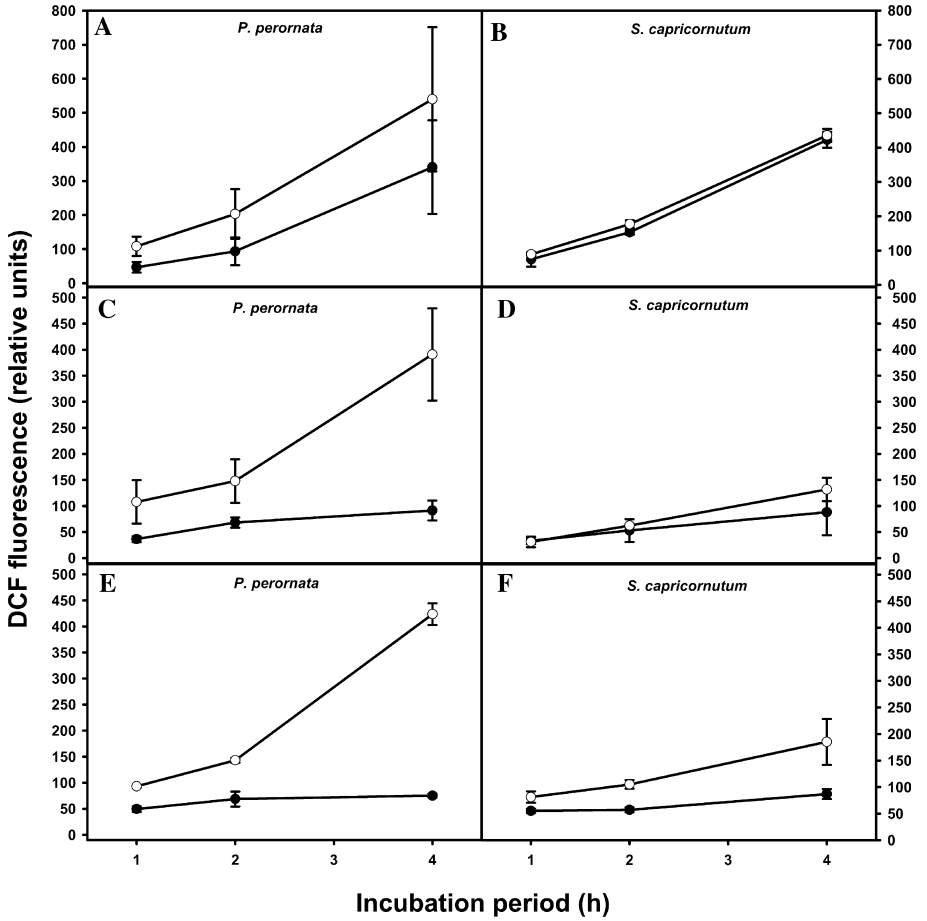

Fig. 5. Effect of 10.0 μM (A,B), 200.0 μM (C,D), and 500.0 μM (E,F) ascorbate (●) on ROS production in cultures of *P. perornata* and *S. capricornutum* containing 1.0 μM WSAD (○). Error bars represent ±1 SD.

α-tocopherol was more effective at reducing ROS production in the cultures at 10.0 μM; however, at the higher concentrations tested, ascorbate appeared more effective since ROS production was almost completely reduced. The experimental method that we used to detect ROS production [12] did not permit accurate measurements after approximately 4 h due to the autooxidation of DCHF even in the dark, so the persistence of the high level of ROS-scavenging by ascorbate could not be determined.

Anthraquinone-2-carboxylate produced similar amounts of ROS compared to controls (data not shown). Previous microplate screening results of anthraquinone-2-carboxylate (unpublished data) revealed an LCIC of 100.0 μM towards *P. perornata*. Therefore, this initial primary breakdown product of WSAD is not very toxic towards *P. perornata* and does not appear to play a role in WSAD toxicity towards *P. perornata*.

Ascorbate at the concentrations tested in the microplate bioassay (0.2, 0.5, and 1.0 μM) did not reduce the toxicity of WSAD (0.01, 0.1, and 0.5 μM) towards *P. perornata* (data not shown). However, α-tocopherol, a more potent ROS-scavenger than ascorbate [17], did reduce the toxicity of 0.5 and 1.0 μM WSAD towards *P. perornata* at 10.0 μM (Fig. 7) and at higher concentrations tested (100.0, 200.0, 500.0, and 1000.0 μM), but not at 0.1 and 1.0 μM α-tocopherol (data not shown). We rea-

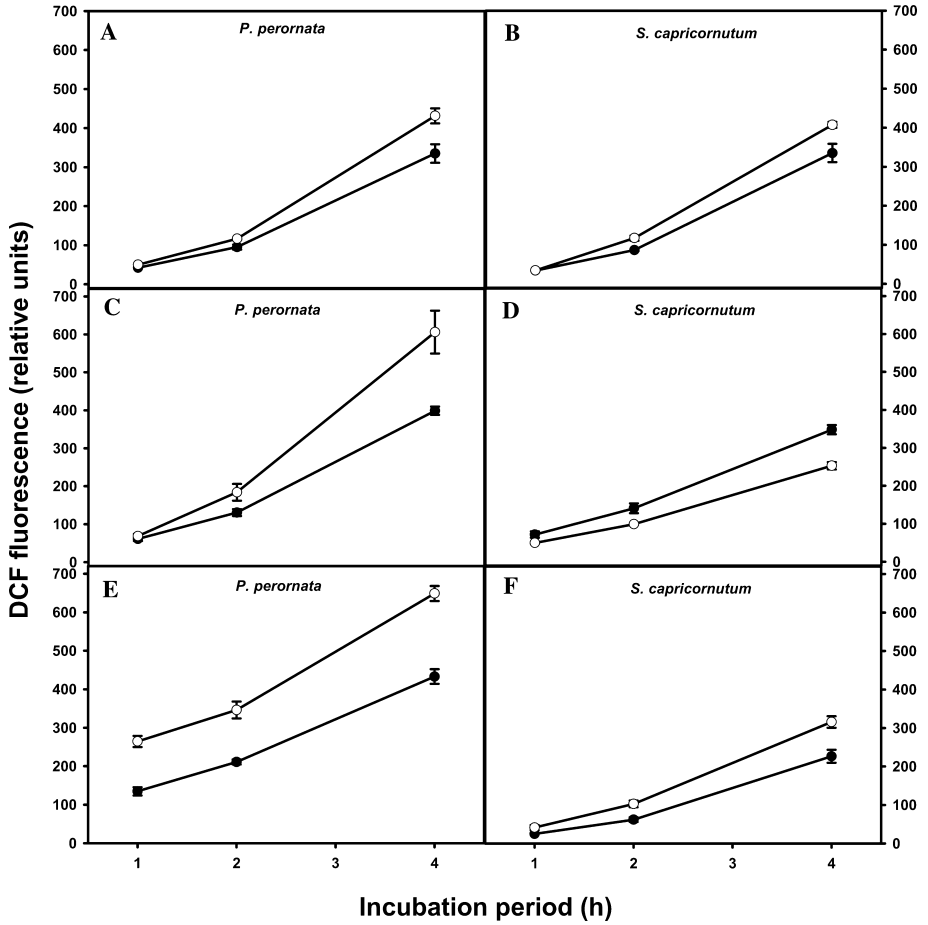

Fig. 6. Effect of 10.0 μM (A,B), 200.0 μM (C,D), and 500.0 μM (E,F) α-tocopherol (●) on ROS production in cultures of *P. perornata* and *S. capricornutum* containing 1.0 μM WSAD (○). Error bars represent ±1 SD.

soned that the ROS-scavenging ability of ascorbate might have been quenched at a faster rate than α-tocopherol. This reversal of the effects of WSAD by α-tocopherol implicates the formation of toxic oxygen species as at least one mode of action of WSAD towards *P. perornata*.

Based upon the results of our study, the toxicity of WSAD against *P. perornata* is related to the generation of ROS. Previous research [9,18] found that other compounds, such as diquat, paraquat, and artemisinin, which generate ROS are very toxic towards *P. perornata* compared to *S. capricornutum*. Quinones can accept electrons, one potential source being photosystem I, and form semiquinone free radicals that subsequently form ROS, e.g., superoxide radicals, hydrogen peroxide, and hydroxyl radicals [19]. Once the semiquinone transfers the single unpaired electron to molecular oxygen, the resulting quinone can be reduced again to re-form a semiquinone, thereby participating in a redox cycle [20]. Subsequently, the generation of ROS in cells can lead to lipid peroxidation, destroying membrane lipids and chlorophyll [15]. The change in the chemical structure of anthraquinone in order to make it water-soluble (i.e., WSAD) appears to be the cause of the different mode of action of WSAD compared to anthraquinone.

If light-induced formation of semiquinones from photosystem I occurs with WSAD, the effi-

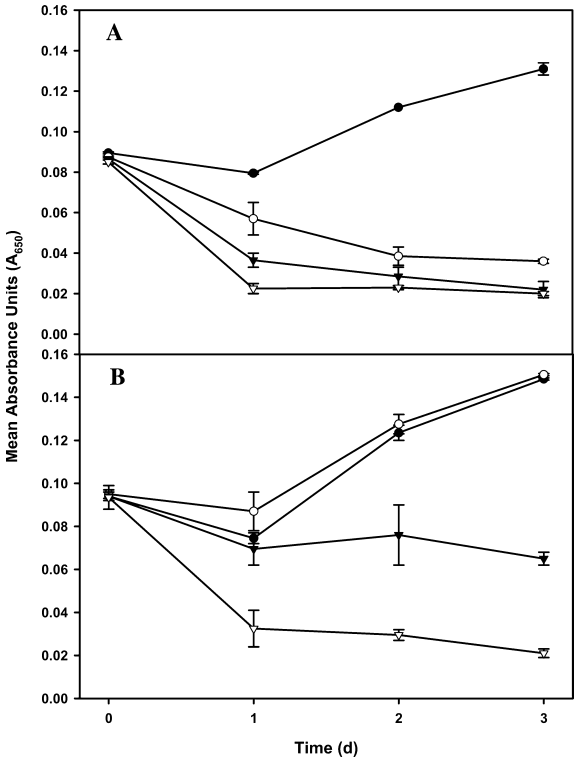

Fig. 7. Effect of no α-tocopherol (A) and 10.0 μM α-tocopherol (B) on the toxicity of 0.5 μM (○), 1.0 μM (▼), and 10.0 μM (▽) WSAD towards *P. perornata*. Controls (●) had no WSAD added. Error bars represent ±1 SD.

cacy of WSAD applied to catfish ponds as a cyanobactericide would be expected to work better on bright, sunny days in which photosynthetic rates would be optimal. Also, most semiquinones are readily reoxidized under aerobic conditions [19], and, therefore, ponds receiving WSAD might need to be mixed continually (e.g., paddlewheel aeration) during the first hours after WSAD application to prevent thermal pond stratification during the daytime and subsequent low dissolved oxygen levels in the hypolimnion [21]. These factors will need to be considered in any future efficacy studies of WSAD performed in catfish ponds.

The selective toxicity of WSAD towards *P. perornata* compared to *S. capricornutum* may be due to a deficiency in the enzyme systems of *P. perornata* that are responsible for scavenging ROS and adapting to oxidative stress. Additional studies need to examine these enzymatic and biochemical activities in *P. perornata*.

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